THE RIBOSOMAL BASIS OF DIAMOND-BLACKFAN ANEMIA: MUTATION AND DATABASE UPDATE

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Key Words:	Diamond-Blackfan anemia, ribosomal protein, erythropoiesis, ribosome biogenesis

THE RIBOSOMAL BASIS OF DIAMOND-BLACKFAN ANEMIA: MUTATION AND DATABASE UPDATE

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ABSTRACT

Diamond-Blackfan Anemia (DBA) is characterized by a defect of erythroid progenitors and, clinically, by anemia and malformations. DBA exhibits an autosomal dominant pattern of inheritance with incomplete penetrance. Currently nine genes, all encoding ribosomal proteins (RP), have been found mutated in approximately 50% of patients. Experimental evidence supports the hypothesis that DBA is primarily the result of defective ribosome synthesis.

By means of a large collaboration among six centers, we report here a mutation update that includes nine genes and 220 distinct mutations, 56 of which are new. The DBA Mutation Database now includes data from 355 patients. Of those where inheritance has been examined, 125 patients carry a *de novo* mutation and 72 an inherited mutation. Mutagenesis may be ascribed to slippage in 65.5% of indels, whereas CpG dinucleotides are involved in 23% of transitions. Using bioinformatic tools we show that gene conversion mechanism is not common in RP genes mutagenesis, notwithstanding the abundance of RP pseudogenes. Genotype-phenotype analysis reveals that malformations are more frequently associated with mutations in *RPL5* and *RPL11* than in the other genes. All currently reported DBA mutations together with their functional and clinical data are included in the DBA Mutation Database.

Key words: Diamond-Blackfan anemia; ribosomal protein; erythropoiesis; ribosome biogenesis.

BACKGROUND

Diamond Blackfan anemia (DBA, OMIM 105650) is a rare inherited disease characterized by severe normochromic macrocytic anemia and reticulocytopenia, typically presenting in the first year of life. Patients generally show a decreased number of erythroid progenitors in their bone marrow [Campagnoli et al., 2004]. The other bone marrow cell lineages are only rarely suppressed. Erythrocytes in DBA patients frequently express fetal hemoglobin (HbF) and erythrocyte adenosine deaminase (eADA) activity, a crucial enzyme of the purine salvage pathway, is elevated in 85% of cases [Glader and Backer, 1988]. DBA is associated with an increased risk of malignancies, especially hematopoietic neoplasms and osteogenic sarcomas [Vlachos et al., 2008]. In 30% to 47% of cases patients show physical malformations involving head, thumb, heart and urogenital system [Lipton, 2006]. Prenatal or postnatal growth retardation independent of steroid therapy is also often present.

The incidence of DBA is around 6 per 1 million live births [Campagnoli et al., 2004]. Most cases are sporadic, but the disease can be inherited with an autosomal dominant pattern. Penetrance is incomplete and expressivity widely variable, even in patients from the same family [Campagnoli et al., 2004]. First-line therapy in DBA patients is steroid treatment. Although 80% of patients have an initial steroid response, less than half the patients can be maintained on a safe and effective dose. Thus many of these initial responders may experience temporary or definitive steroid-resistance of dose limiting toxicity [Vlachos et al., 2008]. Patients who do not respond to steroids undergo chronic blood transfusions and need iron chelation to avoid secondary hemochromatosis. Preliminary data suggest that patients with DBA are more likely to develop iron overload than patients with thalassemia, another disease treated with chronic transfusions [Roggero et al., 2009]. Twenty percent of patients inexplicably achieve remission [Lipton, 2006]. DBA can be treated successfully by allogeneic bone marrow or stem cell transplantation, but the mortality from infections, graft-versus-host

disease and graft failure is significant, especially for unrelated donor transplants [Roy et al., 2005; Vlachos et al., 2008].

The first DBA gene, ribosomal protein (RP) *S19*, was identified in 1999 [Draptchinskaia et al., 1999] and is mutated in about 25% of patients [Willig et al., 1999; Campagnoli et al., 2008]. Mutations in an increasing number of other genes encoding RPs of the small (*RPS24*, *RPS17*, *RPS7*, *RPS10*, *RPS26*) and large (*RPL35A*, *RPL5*, *RPL11*) ribosomal subunits have been recently described in DBA patients [Gazda et al., 2006; Cmejla et al., 2007; Farrar et al., 2008; Gazda et al., 2008; Doherty et al., 2010]. All mutations are present on a single allele, pointing to autosomal dominant inheritance haploinsufficiency. DBA is unquestionably a ribosomapathy, a term initially proposed for dyskeratosis congenita [Luzzatto and Karadimitris, 1998].

In eukaryotes, the ribosome is composed of four different ribosomal RNAs (rRNAs) and 79 ribosomal proteins. While 5S rRNA is transcribed by RNA polymerase III, 28S, 5.8S and 18S rRNAs are processed from a 45S precursor transcribed by RNA polymerase I. The maturation of pre-rRNA occurs in the nucleolus through a complex pathway involving both endo- and exonucleases that remove external and internal transcribed sequences (ETS and ITS). During these steps, the 45S pre-RNA associates with ribosomal proteins, ribonucleases, RNA helicases, small nucleolar RNPs (snoRNPs) and other accessory factors, to form 90S pre-ribosomes. During the maturation process, the 90S pre-ribosome is separated into pre-40S and pre-60S subunits that are exported to the cytoplasm where maturation is completed [Tschochner and Hurt, 2003]. Mature 40S subunits include 18S rRNA and 33 ribosomal proteins whereas mature 60S subunits contain 28S, 5.8S and 5S rRNAs and 46 ribosomal proteins. In humans there are several loci containing rRNA genes, but only one gene for each of the 79 ribosomal proteins.

Molecular mechanisms underlying the causal effect between RP haploinsufficiency and anemia have not been elucidated. A generally recognized pathogenetic hypothesis implies defective ribosome biogenesis leading to apoptosis in erythroid progenitors. This mechanism has been named "ribosomal stress" and there are indications that it may be signalled through p53 [Lipton and Ellis, 2009]. Several RPs have a second function different from their roles as structural components of the ribosome. Defects in these extra-ribosomal functions might also contribute to the overall complexity of DBA phenotypes.

Mutations in DBA genes, along with their functional consequences and genotype-phenotype correlations, have been catalogued in the DBA Mutation Database, created by our group in 2008 and available via www.dbagenes.unito.it [Boria et al., 2008]. Here we report an update of the DBA Mutation Database. The updated database contains nine DBA genes (*RPS19*, *RPS24*, *RPS17*, *RPS7*, *RPS10*, *RPS26*, *RPL5*, *RPL11*, *RPL35A*) and 220 distinct mutations. It now includes information on molecular mechanisms involved in RP mutagenesis and more detailed information about inheritance. This update arises from the collaboration of Czech, French, German, Swedish, American and Italian DBA clinical and research groups.

VARIANTS

RPS19

The *RPS19* gene (OMIM 603474; *locus* 19q13.2) was the first DBA gene that was discovered and is the most frequently mutated in patients. It comprises 6 exons and spans 11 kb. The first exon (372 bp) is not included in the coding DNA sequence (CDS) region, while the other five (435 bp) encode a protein of 145 amino acids (MW ~16 kDa).

Eighty-seven distinct mutations have been previously described in *RPS19* gene [most reviewed in Campagnoli et al., 2008]. We here report 42 additional mutations: 11 missense, 3 nonsense, 18 deletions and/or insertions, 10 splice-site defects (Table 1). Overall 129 distinct

RPS19 mutations are reported and they are carried by 219 patients: 82 of these are *de novo* and 45 are inherited. The inheritance was not ascertained in the remaining cases.

At least 163 polymorphisms are listed in NCBI SNP database. We have identified 9 unpublished intronic allelic variants: c.71+174A>G, c.71+24A>G, c.356+166G>T, c.411+6G>T, c.412-75A>G, c.356+153G>A, c.356+29T>C, c.356+229G>A and c.412-131T>C. Seven pseudogenes are annotated in the NCBI Gene database as 'inferred'.

RPL5

The human *RPL5* gene (OMIM 603634; *locus* 1p22.1) consists of eight exons and spans 9.8 kb. The primary transcript is 1031 nt long and encodes a 297-aa protein (MW ~34.2 kDa) component of the 60S ribosomal subunit.

Heterozygous mutations in *RPL5* gene have been reported in DBA patients [Gazda et al., 2008; Cmejla at al., 2009; Quarello at al., 2010]. Gazda et al. showed mutations in 18 of 196 DBA probands (9%) and in six additional family members [Gazda et al., 2008], for a total of 24 individuals; Cmejla et al. studied 28 Czech families and identified sequence changes in 8 DBA patients from 6 families (21.4%) [Cmejla et al., 2009]; Quarello et al. reported mutations in 12 out of 92 (13%) unrelated Italian probands [Quarello et al., 2010]. In this paper we are adding 10 new mutations found in 12 patients (Table 1) and two patients carrying two previously described mutations. The total number of patients with mutations in *RPL5* is 58: 21 have a *de novo* mutation, 10 are familial cases. Thirty-nine mutations are distinct and are distributed as follows: 6 missense, 7 nonsense, 21 small deletions and/or insertions, 5 splice-site defects.

Ninety-five polymorphisms are listed in NCBI SNP database. There are multiple processed pseudogenes of *RPL5* gene dispersed through the genome: three of them are annotated as 'validated' in NCBI Gene database.

RPL11

The *RPL11* gene is located on chromosome 1 (OMIM 604175; *locus* 1p36.1-p35) and encompasses 6 exons spanning 4.6 kb. The *RPL11* mRNA is 609 bp long and encodes a 178 amino acid protein (MW ~20.1 kDa).

Twenty-three distinct mutations in 34 DBA patients have been previously described [Gazda et al., 2008; Cmejla at al., 2009; Quarello at al., 2010]; three new mutations are reported here for the first time (Table 1). The 26 mutations are classified as follows: 1 missense, 2 nonsense, 17 small deletions and/or insertions, and 6 splice-site defects. The total number of patients with mutations is 37: 12 carry a *de novo* mutation, whereas 7 are familial cases.

Fifty-seven polymorphisms are reported in NCBI SNP database and five pseudogenes are annotated in NCBI Gene database as 'inferred'.

RPL35A

The *RPL35A* gene (OMIM 180468) is located on chromosome 3q29-qter and comprises 6 exons spanning 5.6 kb. The predicted size of the primary transcript is 511 bp. The first exon (41 bp) is not included in the CDS region, while the other five (470 bp) encode a 110-aa protein (MW ~12.4 kDa).

Farrar et al. [2008] reported five mutations: one missense, one nonsense, one small deletion and two deletions of a complete allele. They are all considered pathogenic. The missense mutation c.97G>A, which creates a cryptic splice donor site within exon 3, was also found in the proband's father and sister, both showing isolated macrocytosis. Inheritance was not tested in the remaining cases [Farrar et al., 2008].

The *RPL35A* gene has 45 polymorphisms listed in NCBI SNP database and only one pseudogene annotated as 'validated' in NCBI Gene database.

RPS24

The *RPS24* (OMIM 602412; *locus* 10q22-q23*)* gene comprises 6 exons and spans 8 kb. Its three isoforms are expressed as splice variants: S24a or variant 1 (615 bp), S24c or variant 2

(593 bp) and S24b or variant 3 (633 bp), encoding proteins of 130, 133 and 131 amino acids, respectively. The different isoforms show a tissue-specific pattern of expression [Xu and Roufa, 1996; Gazda et al., 2006].

Three *RPS24* mutations in a total of 8 DBA patients [Gazda et al., 2006] have been previously reported in the DBA Mutation Database. Recently Quarello et al. and Badhai et al. showed two further changes in *RPS24* gene: a small deletion and a missense mutation [Quarello et al., 2010; Badhai et al., 2009]. Additionally, we identified an unpublished splice donor variant (Table 1). In total 6 mutations are reported for this gene: one missense, two nonsense, one small deletion and two splice-site defects. In total, 12 patients were found mutated in this gene: only one has a *de novo* mutation, whereas 5 carry an inherited mutation.

Eighty-two polymorphisms are listed in NCBI SNP database and only one *RPS24* pseudogene is annotated as 'validated' in NCBI Gene database.

RPS17

The *RPS17* gene (OMIM 180472) is located on chromosome 15 (*locus* 15q). It encompasses five exons and spans 3.7 kb. The *RPS17* mRNA is 562 nt long and encodes for a 135-amino acid protein (MW ~15.5 kDa). Two different sequence changes eliminating the natural start site for protein synthesis were found in this gene by Cmejla et al. in 2007 and Song et al. in 2010. Gazda et al. [2008] identified a new mutation among 196 tested probands; it is a deletion of two nucleotides causing a frameshift. All mutations are *de novo*.

The *RPS17* gene has 30 polymorphisms listed in NCBI SNP database and has two 'validated' pseudogenes in NCBI Gene database.

RPS7

The *RPS7* gene (OMIM 603658; *locus* 2p25) consists of 7 exons spanning 5.6 kb. The predicted size of the primary transcript is 745 bp encoding a 194-aa protein (MW ~22 kDa).

A donor splice-site mutation in intron 2 was found in a single DBA patient by Gazda and collaborators [Gazda et al. 2008]. The inheritance was not tested.

Eighty-three polymorphisms are listed in NCBI SNP database for this gene and only one pseudogene is annotated as 'validated' in NCBI Gene database.

RPS26

The *RPS26* gene (OMIM 603701) is located on chromosome 12 (*locus* 12q13) and has four exons spanning 2.32 kb. It results in a transcript of 699 bp encoding a 115-amino acid protein (MW ~12.9 kDa).

Recently, Doherty and collaborators identified 8 distinct mutations in 13 DBA patients: 5 carry a *de novo* mutation, whereas 3 are familial cases [Doherty et al., 2010]. Mutations in this gene were identified in about 6.4% of the overall DBA population and are distributed as follows: 4 missense, one insertion and 3 splice-site defects.

The *RPS26* gene has 64 polymorphisms listed in NCBI SNP database and 4 pseudogenes annotated as 'validated' in NCBI Gene database.

RPS10

The *RPS10* gene (OMIM 603632; *locus* 6p21.31) encompasses 6 exons spanning 8.65 kb. The predicted size of the primary transcript is 636 bp encoding a 165-aa protein (MW ~18.8 kDa). Mutations in this gene were identified in about 2.6% of the overall DBA population [Doherty et al., 2010]. Specifically three distinct sequence changes in 5 DBA probands have been reported: one missense, one nonsense and one insertion. One mutation is *de novo*, whereas in the other cases the inheritance was not ascertained [Doherty et al., 2010].

The *RPS10* gene has 88 polymorphisms reported in NCBI SNP database and 3 'validated' pseudogenes listed in NCBI Gene database.

MUTAGENESIS MECHANISMS

Common mutagenesis mechanisms that generate point mutations include slippage, that causes small indels, and cytosine-guanine (CpG) dinucleotide methylation followed by spontaneous deamination, which causes G>A or C>T transitions. We evaluated the involvement of these mechanisms in DBA by comparing each mutant sequence to the wild-type gene sequence and by observing the context in which a mutation occurred. We found that 57 out of 87 indels in DBA patients are consistent with a slippage mechanism. The frequency of the different substitution classes is 64 transitions versus 50 transversions. Out of 64 transitions, we identified 15 mutations occurring within CpG dinucleotides.

A rare mechanism of mutagenesis is interlocus gene conversion arising from transfer of genetic information between highly homologous genes. Well-known diseases caused by this process are steroid 21-hydroxylase deficiency [Morel et al., 1989] and Shwachman-Diamond syndrome [Boocock et al., 2003]. In these cases the donor sequence is a nearby pseudogene resulting from a recent duplication. To investigate the occurrence of pseudogene-mediated gene conversion in DBA, we retrieved pseudogene sequences of the most frequently mutated RPs (*RPS19*, *RPL5* and *RPL11*) annotated as 'validated' and/or 'inferred' in NCBI Gene database; all of them were intronless. We aligned them with their respective RP gene sequences and looked at 5bp on each side of the mutation, searching for a correspondence between the pseudogene and the mutated sequence. We found only 6 mutations possibly due to gene conversion: 4 in *RPS19*, 1 in *RPL5* and 1 in *RPL11* (Table 2). The pseudogenes are all located on different chromosomes, as compared to the respective genes. Two mutations in *RPS19* are identical to the corresponding sequence of pseudogene *RPS19P2* located on chromosome 1. Obviously, we could not exclude that the same changes arose independently in the gene and in the pseudogene. In any case, our results show that gene conversion does not play a major role in generating mutations in *RPS19*, *RPL5* and *RPL11*.

In conclusion, the most frequent mutagenic mechanism observed in DBA patients appears to be slippage, followed by transitions occurring at CpG dinucleotides.

BIOLOGICAL RELEVANCE

Several studies have addressed the effects of DBA mutations showing that they can lead either to a reduction of RP mRNA or to the production of ribosomal proteins with defective stability and/or localization. In all these cases, mutations cause haploinsufficiency which in turn interferes with the biogenesis of the large or the small ribosomal subunit. These defects are due to aberrant rRNA maturation at different steps, depending on the affected RP [Flygare et al., 2007; Choesmel et al., 2007; Idol et al., 2008; Gazda et al., 2008; Farrar et al., 2008; Doherty et al., 2010]. Here, we briefly revisit the biological function of each DBA RP and the effects of their mutations.

RPS19. RPS19 mutations are associated with a defect in the maturation of 18S rRNA resulting in the accumulation of 21S pre-rRNA precursors [Flygare et al., 2007; Choesmel et al., 2007; Idol et al., 2008]. All of these mutations cause loss of function and some have been functionally characterized. Extensive functional data were recently reviewed by Campagnoli et al. in this journal [Campagnoli et al., 2008]. Furthermore Crétien and collaborators reported the study of the subcellular localization of several RPS19 mutants fused to green fluorescent protein (GFP) [Crétien et al., 2008]. They observed impaired nucleolar localization and a marked decrease in levels of protein expression for the following mutants: p.Leu131Pro, p.Trp33X, p.Tyr48X, p.Arg56X, p.Met75X, p.Arg94X, p.Glu13ArgfsX17, p.Arg82ThrfsX72, p.Leu131GlyfsX22. In contrast, p.Trp52Cys, p.Val9_Phe14del and p.Gly120Ser mutants exhibited normal expression and localization. Proteasome inhibitors improved both the expression level and the nucleolar localization of p.Val15Phe, p.Gly127Glu, p.Leu131Pro, p.Arg94X, p.Arg82ThrfsX72 and p.Leu131GlyfsX22 mutants, but had no effect on

p.Glu13ArgfsX17, p.Trp33X, p.Tyr48X, p.Arg56X, and p.Met75X RPS19 proteins [Crétien et al., 2008]. Another mutation was recently investigated for its functional consequences by Badhai and collaborators, who reported that primary fibroblasts from a DBA patient with a *RPS19* acceptor splice-site mutation (c.72-2A>C) showed reduced proliferative capacity due to G1 arrest [Badhai et al., 2009].

RPL5. RPL5 has been implicated in nucleocytoplasmic transport of 5S rRNA prior to its assembly into the large ribosomal subunit [Steitz et al., 1988]. RPL5 specifically binds to this rRNA through the domains located at both the amino terminus and the carboxyl terminus [Michael and Dreyfuss, 1996]. It has been shown that the perturbation of ribosomal biogenesis by impaired rRNA synthesis, processing, or ribosome assembly, triggers the direct binding of RPL5 along with RPL11 and possibly RPL23 to MDM2. These interactions inhibit MDM2-mediated p53 ubiquitination and degradation, resulting in p53 activation [Zhang and Lu, 2009]. The pathogenic effect of *RPL5* haploinsufficiency on ribosome biogenesis has been studied by Gazda and collaborators both in a knockdown cell model and in patient cells that harbored the following mutations: c.67C>T, c.173delG, c.175_176delGA, c.[498_502delTGTGG;497_498ins40] [Gazda et al., 2008]. HeLa cells expressing small interfering RNAs (siRNAs) against *RPL5* show decreased production of 28S and 5.8S mature rRNAs and accumulation of their precursors, in particular 32S and 12S. The same defect was observed in lymphoblastoid cells established from DBA patients. Moreover, RPL5 knockdown induces reduction of free 60S subunit and formation of half-mer polysomes [Gazda et al., 2008].

RPL11. In yeast, Rpl11 forms a subcomplex with Rpl5 and 5S rRNA that is recruited into nascent ribosomes at an early step [Zhang et al., 2007]. In human cells, RPL11 appears to have a similar role in ribosome biogenesis but also functions to suppress the transcriptional activity of c-Myc and plays a feedback regulatory role in coordinating c-Myc level and

activity with ribosomal biogenesis [Dai et al., 2007]. RPL11 also cooperates with RPL5 to inhibit the E3 ubiquitin ligase activity of MDM2, thus resulting in the accumulation of transcriptionally active p53 [Zhang et al., 2003]. Fumagalli et al. recently showed that RPL11-mediated p53 induction is a general response to defective 40S or 60S ribosome biogenesis in human cell lines [Fumagalli et al. 2009]. The ubiquitin-like molecule NEDD8, that controls RPL11 stability and subcellular localization, plays an important role in the regulation of RPL11 signaling to p53 [Sundqvist et al., 2009]. In zebrafish rpl11 knockdown activates the p53 pathway and disrupts the normal embryonic development through a p53-mediated apoptotic response [Chakraborty et al., 2009]. Gazda and collaborators showed that mutations in *RPL11*, c.314_315delTT, IVS1+2t>c, IVS2-1g>a, IVS4+1g>t, lead to accumulation of the precursors of 28S and 5.8S rRNAs, similar to that of mutations in *RPL5* [Gazda et al., 2008].

RPL35A. Farrar et al. studied RPL35A deficiency in UT-7/Epo and TF-1 cells by transduction with three different small hairpin RNAs (shRNAs) against Rpl35A mRNA. They observed decreased proliferation, increased apoptosis and reduced biogenesis of 60S subunits. Metabolic rRNA labeling and Northern blot analysis revealed accumulation of 45S and 41S early precursors and decreased 12S and 7S pre-RNAs. An EBV-transformed lymphoblastoid cell line from a DBA patient with deletion of a complete allele also showed reduced 12S rRNA compared to healthy controls [Farrar et al., 2008].

RPS24. RPS24, like RPS19, is essential for the production of the small ribosomal subunit, as displayed by the reduction of 40S subunits and 80S monosomes in polysomal profiles of RPS24-depleted cells [Choesmel et al., 2008]. Lymphoblastoid cells from three patients with mutations in RPS24 (p.Gln106X, p.Arg16X, deletion N2-Q22) showed delayed maturation of 30S pre-rRNA with a corresponding decrease in 21S and 18S-E pre-rRNAs. Accumulation of the 30S pre-rRNA suggests that RPS24 is required for the maturation of both the 5′ and 3′

ends of 18S rRNA. Primary fibroblasts obtained from a DBA patient with an *RPS24* start codon mutation (c.1A>G) showed reduced proliferation and abnormal expression of cell cycle regulatory proteins [Badhai et al., 2009]. Moreover, Quarello et al. expressed FLAG-tagged RPS24 protein carrying the mutation p.Gln22del in HEK293 cells to study its subcellular localization. Although this mutant protein is less stable than the wild-type, it was able to reach the nucleolus [Quarello et al., 2010].

RPS7. RPS7, like other RPs discussed above, interacts with MDM2 and regulates its E3 ligase activity on p53 [Chen et al., 2007]. RPS7 is itself a substrate of MDM2 and RPS7 ubiquitination enhances p53 response and facilitates cell death triggered by different stress signals [Zhu et al., 2009]. The pathogenic effect of the *RPS7* mutation c.147+1G>A was studied in lymphoblastoid cells derived from the one patient. These cells show accumulation of 45S and 30S pre-rRNAs when compared to an unaffected sibling. Depletion of RPS7 by siRNA in HeLa cells confirmed a defect in 5'-ETS processing [Gazda et al., 2008].

RPS26. RPS26 can regulate its own expression by binding its pre-mRNA and suppressing its splicing [Ivanov et al., 2005]. Northern blot analysis showed that depletion of RPS26 in HeLa cells leads to the accumulation of 43S, 26S and 18S-E pre-rRNAs, pointing to defective cleavage at both ends of 18S. The same phenotype was present in lymphoblastoid cells derived from RPS26 mutated patients (c.1A>T, c.1A>G in two different probands, c.97G>A and IVS1+1g>c) [Doherty et al., 2010].

RPS10. Analysis of RPS10 depleted HeLa cells and RPS10 mutated lymphoblastoid cells (c.260_261insC and c.337C>T in three different probands) revealed a pre-rRNA processing phenotype similar to RPS26 [Doherty et al., 2010].

CLINICAL RELEVANCE

For many years DBA was considered to be rarely inherited since most patients presented without any family history. Mutational analyses have clarified this observation: in our study, 125 of 197 patients whose family history was ascertained had de novo mutations, whereas the other 72 were familial. Thus, it is likely that the majority of DBA mutations arise spontaneously. This pattern may be due to reduced reproductive fitness of mutated patients. Difficulties in completing pregnancy and an increase in stillborn offspring have been reported for those women with DBA who have conceived [Faivre et al., 2006].

Genotype-Phenotype Correlation

Genotype-phenotype correlations were evaluated in all patients reported in the DBA Mutation Database. They represent approximately 50% of the total number of DBA patients. Clinical description was not available for a few patients thus each analysis was calculated only for patients that had the specific clinical information.

The statistical analysis was performed by considering the following parameters: growth retardation, craniofacial malformation (other than cleft lip and palate), cleft lip and/or palate, upper limb abnormalities, flat thenar muscle, malformed thumb, triphalangeal thumb, heart anomalies, genitourinary anomalies, presence of any type of malformation including short stature, any type of malformation with the exclusion of short stature, multiple malformations, mental retardation, small for gestational age (SGA), response to steroids (Table 3). Hematological information was not considered because clinical data reported in old publications were not updated. The risk connected with carrying a mutation in a specific RP relative to patients carrying mutations in the other eight RP genes studied was obtained by using logistic regression and odds ratio (OR) and 95% CI were calculated. P values less than 0.05 were considered statistically significant. Data were analyzed with the SAS software

version 8.01. Data were not informative for some rarely mutated RP genes, namely *RPS7*, *RPS17*, *RPS24* and *RPS10* (Table 3). Results for *RPS19*, *RPL5* and *RPL11* are presented in Fig. 1.

As previously shown [Gazda et al., 2008; Cmejla at al., 2009; Quarello at al., 2010], patients carrying mutations in *RPL5* or *RPL11* present more frequently with malformations. The risk of malformations of any type, including or excluding short stature, is 6.5 or 7.6-fold higher in RPL5-mutated and 4.5 or 2.7-fold higher in RPL11-mutated patients, respectively, than in patients with mutations in other genes. RPL5-mutated patients have a statistically higher risk of multiple malformations (OR 3.8). Each type of malformation evaluated in this study, with the exception of those of the genitourinary tract, is more frequent in patients with mutations in *RPL5*. Specifically, 21 out of 24 patients with cleft have mutations in *RPL5*. A cleft was shown in two patients who carried mutations in *RPL11* and in one patient who carried a mutation in *RPS26*. Clefts have never been found in *RPS19* patients. RPL5-mutated patients are SGA more frequently than patients with other mutations. Of 9 patients with SGA, 7 carry a *RPL5* and 2 a *RPL11* mutation. Although mutations in *RPL11* are associated with an increased risk of any type of malformations, most of these are hand abnormalities (Table 3).

The gene currently associated with genitourinary malformations is *RPL35A*. Of 5 patients with mutations in *RPL35A*, 3 have genitourinary malformations. In contrast, only 16 of 249 patients with mutations in other RP genes have genitourinary malformations (Table 3). This difference in frequencies towards is statistically significant. Conversely, patients with mutations in *RPS19* are less likely to have malformations of any type when compared with the other patients (OR < 1). This finding is also true when each type of malformation is considered independently.

Patients mutated in *RPS26* exhibit the lowest response to steroids (4 out of 10), whereas most DBA patients with mutations in other known RP genes respond to steroids at a higher frequency (92/125) (CI 0.06-0.90).

Interestingly, mental retardation is shown in only 9/270 patients. Eight of these have mutations in *RPS19*: four have large deletions at the *RPS19* locus, two have translocations associated with deletions, one has a deletion of exons 1-3 and one has a splice-site defect (c.72-1G>A). The last patient has a frameshift mutation in *RPL5* (c.169_172delAACA) and a complex malformation phenotype that includes myelomeningocele, cleft palate, and facial dysmorphism. Patients with mental retardation and large deletions/rearrangements in *RPS19* are likely to show a contiguous gene syndrome [Tentler et al., 2000]. In conclusion, we can say that mental retardation is not typically associated with mutations in ribosomal protein genes and when found in association with other clinical features of DBA is probably linked to contiguous genes.

Variable expressivity

Variable expressivity is shown for all RP gene mutations. Possible mechanisms underlying variable expressivity include an influence of modifier genes and environmental factors.

Stochastic factors are invoked in the case of non concordance for malformations in monozygous twins [Campagnoli et al., 2004]. Potential modifier genes could be genes involved in modulating the level of expression of RP genes or other genes involved in ribosome biogenesis. A patient harboring mutations in two different RP genes, *RPL5* and *RPS24*, was reported by Quarello et al. (2010). In this case, the malformation phenotype was likely due to *RPL5*, since the patient carried hand malformation, often associated with *RPL5* mutations. Moreover *RPL5* mutation was *de novo* and the parent carrier of the *RPS24* missense mutation did not show malformations. These aspects suggest that the *RPS24*

missense variant may be a silent mutation. Variations in the promoter or other regions have also been hypothesized to be phenotype modifiers [Crétien et al., 2010].

DATABASE

The need for a comprehensive collection of all mutations in DBA genes, as well as of their functional consequences and clinical phenotypes, prompted us to create and maintain the DBA Mutation Database (http://www.dbagenes.unito.it) [Boria et al. 2008]. It is founded on the Leiden Open (source) Variation Database (LOVD) system that was upgraded to the latest version, LOVD 2.0 build 25, released in March 2010 [Fokkema et al., 2005].

The first version of the database included only three DBA genes, *RPS19*, *RPS24* and *RPS17*, and 86 distinct disease-causing mutations. The database has been updated with the newly described DBA genes: *RPL5*, *RPL11*, *RPL35A*, *RPS7*, *RPS26*, *RPS10*, and now comprises a total of 220 distinct pathogenetic mutations, distributed as follows: 52 missense, 27 nonsense, 87 small deletions and insertions, 14 large deletion and rearrangements and 40 splice-site defects (Table 4). Out of the 134 newly added sequence changes, 78 were previously published while 56 are reported here for the first time. Overall, the database includes data for 355 patients, all carrying RP mutations.

According to the basic structure of the LOVD database scheme, each DBA gene has its own homepage providing general gene and database information, access to allelic variant tables, search tools for browsing data and links to external gene-related resources, such as NCBI SNP database, MIM, NCBI Entrez, HGMD (Supp. Figure S1). Furthermore links to schematic drawings showing the location of the pathogenetic variants in relation to the gene (Supp. Figure S2) and, when available, to the protein structures are included.

All mutations are described according to the Human Variation Society (HGVS) nomenclature [den Dunnen and Antonarakis 2000] and their pathogenicity was established according to the

HUGO Mutation Database Initiative/HGVS (Supplemental Material). Mutation nomenclature has been checked with Mutalyzer program [Wildeman et al., 2008].

All available data relative to each mutation are provided in the "Variants" section and include the exact molecular description at DNA and protein levels, the clinical features of the corresponding patients, literature references and details on the detection methods. Consistent with the functional classification proposed for RPS19 by Campagnoli et al. (2008), information about functional consequences of mutations on mRNA and protein are reported.

Compared to the previous database version, it is now possible to specify the potential molecular mechanism leading to each allelic variant ("Molecular Mechanisms" column) and for each patient the clinical complications ("Complications" column). Furthermore, we substituted the "Occurrence" column with "Variant Origin" that describes the inheritance of the mutation in an exhaustive way. Further details can be found at the database web site.

A link to the DBA Mutation Database is provided for each gene in NCBI Gene database.

DIAGNOSTIC RELEVANCE

At the time of submission, 220 distinct mutations in 355 DBA patients have been identified in nine genes, all encoding ribosomal proteins. The difficulties in clinical diagnosis and the absence of biochemical assays make identification of the causative mutation clinically important. Identification of non-symptomatic carriers is mandatory when potential donors of hematological stem cells are evaluated within first degree relatives. Moreover, prenatal diagnosis may be requested by families with severely affected children. The *RPS19* gene is the most frequently involved being mutated in 25% of patients. Mutations in *RPL5* and *RPL11* are frequently found in patients with malformations of upper limbs or face. Malformations in general, also appear increased in patients with mutations in these genes. A

patient with these types of malformations or with multiple malformations should be screened first for these genes.

FUTURE PROSPECTS

The ribosomal basis of DBA is evident. So far about 50% of DBA patients may be characterized using the four most commonly mutated genes: *RPS19*, *RPL5*, *RPL11*, *RPS26*. It is expected that other RP genes may be mutated in the remaining patients. For this reason the DBA community has started a large project focused at sequencing each of the 79 RP genes in every DBA patient.

However, sequencing is tedious and time-consuming. An easy and quick diagnostic assay would be of great help to clinical hematologists: the perfect assay should be able to diagnose all DBA patients, independently of the gene affected. A genetic or functional abnormality shared by all patients may be exploited to generate a diagnostic assay.

The definition of the molecular basis of DBA has also opened the road to molecular therapy. Gene therapy looks promising since even a small increase of RP expression may be helpful to resolve the bone marrow failure thereby making this disease a reasonable target for this treatment [Flygare et al., 2008]. Treatment with leucine has been proven helpful in rare cases [Pospisilova et al., 2007] but large clinical trials are necessary to ascertain if its effect may be general or gene specific.

Accession Numbers

The GenBank accession number for human RPs are: NM_001022.3 for *RPS19*, NM_000969.3 for *RPL5*, NM_000975.2 for *RPL11*, NM_000996.2 for *RPL35A*, NM_033022.3 for *RPS24*, NM_001021.3 for *RPS17*, NM_001011.3 for *RPS7*, NM_001029.3 for *RPS26* and NM_001014.3 for *RPS10*.

Web Resources

The URLs of resources cited in this work are the following:

Single Nucleotide Polymorphisms database, http://www.ncbi.nlm.nih.gov/SNP/

Entrez Gene database, http://www.ncbi.nlm.nih.gov/gene/

Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/omim

The Human Gene Mutation Database, http://www.hgmd.cf.ac.uk

PolyPhen, http://genetics.bwh.harvard.edu/pph/

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REFERENCES

Badhai J, Froejmark AS, Davey EJ, Schuster J, Dahl N. 2009. Ribosomal protein S19 and S24 insufficiency cause distinct cell cycle defects in Diamond-Blackfan anemia. Biochim Biophys Acta 1792:1036-42.

Boocock GR, Morrison JA, Popovic M, Richards N, Ellis L, Durie PR, Rommens JM. 2003. Mutations in SBDS are associated with Shwachman-Diamond syndrome. Nat Genet 33:97-101.

Boria I, Quarello P, Avondo F, Garelli E, Aspesi A, Carando A, Campagnoli MF, Dianzani I, Ramenghi U. 2008. A new database for ribosomal protein genes which are mutated in Diamond-Blackfan Anemia. Hum Mutat 29:E263-70.

Campagnoli MF, Garelli E, Quarello P, Carando A, Varotto S, Nobili B, Longoni D, Pecile V, Zecca M, Dufour C, Ramenghi U, Dianzani I. 2004. Molecular basis of Diamond-Blackfan anemia: new findings from the Italian registry and a review of the literature. Haematologica 89:480-489.

Campagnoli MF, Ramenghi U, Armiraglio M, Quarello P, Garelli E, Carando A, Avondo F, Pavesi E, Fribourg S, Gleizes PE, Loreni F, Dianzani I. 2008. RPS19 mutations in patients with Diamond-Blackfan anemia. Hum Mutat 29:911-20.

Chakraborty A, Uechi T, Higa S, Torihara H, Kenmochi N. 2009. Loss of ribosomal protein L11 affects zebrafish embryonic development through a p53-dependent apoptotic response. PLoS One 4:e4152.

Chen D, Zhang Z, Li M, Wang W, Li Y, Rayburn ER, Hill DL, Wang H, Zhang R. 2007. Ribosomal protein S7 as a novel modulator of p53-MDM2 interaction: binding to MDM2, stabilization of p53 protein, and activation of p53 function. Oncogene 26:5029-37.

Choesmel V, Bacqueville D, Rouquette J, Noaillac-Depeyre J, Fribourg S, Crétien A, Leblanc T, Tchernia G, Da Costa L, Gleizes PE. 2007. Impaired ribosome biogenesis in Diamond-Blackfan anemia. Blood 109:1275-83.
Choesmel V, Fribourg S, Aguissa-Touré AH, Pinaud N, Legrand P, Gazda HT, Gleizes PE. 2008. Mutation of ribosomal protein RPS24 in Diamond-Blackfan anemia results in a ribosome biogenesis disorder. Hum Mol Genet 17:1253-63.

Cmejla R, Cmejlova J, Handrkova H, Petrak J, Pospisilova D. 2007. Ribosomal protein S17 gene (RPS17) is mutated in Diamond-Blackfan anemia. Hum Mutat 28:1178-1182.

Cmejla R, Cmejlova J, Handrkova H, Petrak J, Petrtylova K, Mihal V, Stary J, Cerna Z, Jabali Y, Pospisilova D. 2009. Identification of mutations in the ribosomal protein L5 (RPL5) and ribosomal protein L11 (RPL11) genes in Czech patients with Diamond-Blackfan anemia. Hum Mutat 30:321-7.

Crétien A, Hurtaud C, Moniz H, Proust A, Marie I, Wagner-Ballon O, Choesmel V, Gleizes PE, Leblanc T, Delaunay J, Tchernia G, Mohandas N, Da Costa L. 2008. Study of the effects

of proteasome inhibitors on ribosomal protein S19 (RPS19) mutants, identified in patients with Diamond-Blackfan anemia. Haematologica 93:1627-34.

Crétien A, Proust A, Delaunay J, Rincé P, Leblanc T, Ducrocq R, Simansour M, Marie I, Tamary H, Meerpohl J, Niemeyer C, Gazda H, Sieff C, Ball S, Tchernia G, Mohandas N, Da Costa L. 2010. Genetic variants in the noncoding region of RPS19 gene in Diamond-Blackfan anemia: potential implications for phenotypic heterogeneity. Am J Hematol 85:111-6.

Dai MS, Sears R, Lu H. Feedback regulation of c-Myc by ribosomal protein L11. 2007. Cell Cycle 6:2735-41.

den Dunnen JT, Antonarakis SE. 2000. Mutation nomenclature extensions and suggestions to describe complex mutations: a discussion. Hum Mutat. 15:7-12.

Doherty L, Sheen MR, Vlachos A, Choesmel V, O'Donohue MF, Clinton C, Schneider HE, Sieff CA, Newburger PE, Ball SE, Niewiadomska E, Matysiak M, Glader B, Arceci RJ, Farrar JE, Atsidaftos E, Lipton JM, Gleizes PE, Gazda HT. 2010. Ribosomal protein genes RPS10 and RPS26 are commonly mutated in Diamond-Blackfan anemia. Am J Hum Genet. 86:222-8.

Faivre L, Meerpohl J, Da Costa L, Marie I, Nouvel C, Gnekow A, Bender-Götze C, Bauters F, Coiffier B, Peaud PY, Rispal P, Berrebi A, Berger C, Flesch M, Sagot P, Varet B, Niemeyer C, Tchernia G, Leblanc T. 2006. High-risk pregnancies in Diamond-Blackfan anemia: a survey of 64 pregnancies from the French and German registries. Haematologica 91:530-3.

Farrar JE, Nater M, Caywood E, McDevitt MA, Kowalski J, Takemoto CM, Talbot CC Jr, Meltzer P, Esposito D, Beggs AH, Schneider HE, Grabowska A, Ball SE, Niewiadomska E, Sieff CA, Vlachos A, Atsidaftos E, Ellis SR, Lipton JM, Gazda HT, Arceci RJ. 2008. Abnormalities of the large ribosomal subunit protein, Rpl35a, in Diamond-Blackfan anemia. Blood 112:1582-92.

Flygare J, Aspesi A, Bailey JC, Miyake K, Caffrey JM, Karlsson S, Ellis SR. 2007. Human RPS19, the gene mutated in Diamond-Blackfan anemia, encodes a ribosomal protein required for the maturation of 40S ribosomal subunits. Blood 109:980-6.

Flygare J, Olsson K, Richter J, Karlsson S. 2008. Gene therapy of Diamond Blackfan anemia CD34(+) cells leads to improved erythroid development and engraftment following transplantation. Exp Hematol 36:1428-35.

Fokkema IF, den Dunnen JT, Taschner PE. 2005. LOVD: easy creation of a locus-specific sequence variation database using an "LSDB-in-a-box" approach. Hum Mutat 26:63-8.

Fumagalli S, Di Cara A, Neb-Gulati A, Natt F, Schwemberger S, Hall J, Babcock GF, Bernardi R, Pandolfi PP, Thomas G. 2009. Absence of nucleolar disruption after impairment of 40S ribosome biogenesis reveals an rpL11-translation-dependent mechanism of p53 induction. Nat Cell Biol 11:501-8.

Gazda HT, Grabowska A, Merida-Long LB, Latawiec E, Schneider HE, Lipton JM, Vlachos A, Atsidaftos E, Ball SE, Orfali KA, Niewiadomska E, Da Costa L, Tchernia G, Niemeyer C,

Meerpohl JJ, Stahl J, Schratt G, Glader B, Backer K, Wong C, Nathan DG, Beggs AH, Sieff CA. 2006. Ribosomal protein S24 is mutated in Diamond-Blackfan anemia. Am J Hum Genet 79:1110-1118.

Gazda HT, Sheen MR, Vlachos A, Choesmel V, O'Donohue MF, Schneider H, Darras N, Hasman C, Sieff CA, Newburger PE, Ball SE, Niewiadomska E, Matysiak M, Zaucha JM, Glader B, Niemeyer C, Meerpohl JJ, Atsidaftos E, Lipton JM, Gleizes PE, Beggs AH. 2008. Ribosomal protein L5 and L11 mutations are associated with cleft palate and abnormal thumbs in Diamond-Blackfan anemia patients. Am J Hum Genet 83:769-80.

Glader BE, Backer K. 1988. Elevated red cell adenosine deaminase activity: a marker of disordered erythropoiesis in Diamond-Blackfan anaemia and other haematologic diseases. Br J Haematol 68:165-168.

Idol RA, Robledo S, Du HY, Crimmins DL, Wilson DB, Ladenson JH, Bessler M, Mason PJ. 2007. Cells depleted for RPS19, a protein associated with Diamond Blackfan Anemia, show defects in 18S ribosomal RNA synthesis and small ribosomal subunit production. Blood Cells Mol Dis 39:35-43.

Ivanov AV, Malygin AA, Karpova GG. 2005. Human ribosomal protein S26 suppresses the splicing of its pre-mRNA. Biochim Biophys Acta 1727:134-40.

Lipton JM. 2006. Diamond Blackfan anemia: new paradigms for a “not so pure” inherited red cell aplasia. Semin Hematol 43:167-177.

Lipton JM and Ellis SR. 2009. Diamond-Blackfan anemia: diagnosis, treatment, and molecular pathogenesis. Hematol Oncol Clin North Am 23:261-82.

Luzzatto L, Karadimitris A. 1998. Dyskeratosis and ribosomal rebellion. Nat Genet 19:6-7.

Michael WM and Dreyfuss G. 1996. Distinct domains in ribosomal protein L5 mediate 5 S rRNA binding and nucleolar localization. J Biol Chem 271:11571-4.

Morel Y, David M, Forest MG, Betuel H, Hauptman G, Andre J, Bertrand J, Miller WL. 1989. Gene conversions and rearrangements cause discordance between inheritance of forms of 21-hydroxylase deficiency and HLA types. J Clin Endocrinol Metab 68:592-9.

Pospisilova D, Cmejlova J, Hak J, Adam T, Cmejla R. 2007. Successful treatment of a Diamond-Blackfan anemia patient with amino acid leucine. Haematologica 92:e66-e67.

Proust A, Da Costa L, Rince P, Landois A, Tamary H, Zaizov R, Tchernia G, Delaunay J; SHIP Working Group on DBA. 2003. Ten novel Diamond-Blackfan anemia mutations and three polymorphisms within the rps19 gene. Hematol J. 4:132-6.

Quarello P, Garelli E, Carando A, Brusco A, Calabrese R, Dufour C, Longoni D, Misuraca A, Vinti L, Aspesi A, Biondini L, Loreni F, Dianzani I, Ramenghi U. 2010. Diamond-Blackfan anemia: genotype-phenotype correlation in Italian patients with RPL5 and RPL11 mutations. Haematologica 95:206-13.

Roggero S, Quarello P, Vinciguerra T, Longo F, Piga A, Ramenghi U. 2009. Severe iron overload in Blackfan-Diamond anemia: a case-control study. Am J Hematol 84:729-32.

Roy V, Pérez WS, Eapen M, Marsh JCW, Pasquini M, Pasquini R, Mustafa MM, Bredeson CN. 2005. Bone marrow transplantation for Diamond-Blackfan anemia. Biol Blood Marrow Transplant 11:600-608.

Song MJ, Yoo EH, Lee KO, Kim GN, Kim HJ, Kim SY, Kim SH. 2010. A novel initiation codon mutation in the ribosomal protein S17 gene (RPS17) in a patient with Diamond-Blackfan anemia. Pediatr Blood Cancer 54:629-31.

Steitz JA, Berg C, Hendrick JP, La Branche-Chabot H, Metspalu A, Rinke J, Yario T. 1988. A 5S rRNA/L5 complex is a precursor to ribosome assembly in mammalian cells. J Cell Biol 106:545-56.

Sundqvist A, Liu G, Mirsaliotis A, Xirodimas DP. 2009. Regulation of nucleolar signalling to p53 through NEDDylation of L11. EMBO Rep 10:1132-9.

Tentler D, Gustavsson P, Elinder G, Eklöf O, Gordon L, Mandel A, Dahl N. 2000. A microdeletion in 19q13.2 associated with mental retardation, skeletal malformations, and Diamond-Blackfan anaemia suggests a novel contiguous gene syndrome. J Med Genet 37:128-31.

Tschochner H, Hurt E. 2003. Pre-ribosomes on the road from the nucleolus to the cytoplasm. Trends Cell Biol 13:255-263.

Vlachos A, Ball S, Dahl N, Alter BP, Sheth S, Ramenghi U, Meerpohl J, Karlsson S, Liu JM, Leblanc T, Paley C, Kang EM, Leder EJ, Atsidaftos E, Shimamura A, Bessler M, Glader B, Lipton JM; Participants of Sixth Annual Daniella Maria Arturi International Consensus Conference. 2008. Diagnosing and treating Diamond Blackfan anaemia: results of an international clinical consensus conference. Br J Haematol 142:859-76.

Welch EM, Barton ER, Zhuo J, Tomizawa Y, Friesen WJ, Trifillis P, Paushkin S, Patel M, Trotta CR, Hwang S, Wilde RG, Karp G, Takasugi J, Chen G, Jones S, Ren H, Moon YC, Corson D, Turpoff AA, Campbell JA, Conn MM, Khan A, Almstead NG, Hedrick J, Mollin A, Risher N, Weetall M, Yeh S, Branstrom AA, Colacino JM, Babiak J, Ju WD, Hirawat S, Northcutt VJ, Miller LL, Spatrick P, He F, Kawana M, Feng H, Jacobson A, Peltz SW, Sweeney HL. 2007. PTC124 targets genetic disorders caused by nonsense mutations. Nature 447:87-91.

Wildeman M, van Ophuizen E, den Dunnen JT, Taschner PEM. 2008. Improving sequence variant descriptions in mutation databases and literature using the Mutalyzer sequence variation nomenclature checker. Hum Mutat 29:6-13.

Willig TN, Draptchinskaia N, Dianzani I, Ball S, Niemeyer C, Ramenghi U, Orfali K, Gustavsson P, Garelli E, Brusco A, Tiemann C, Pérignon JL, Bouchier C, Cicchiello L, Dahl N, Mohandas N, Tchernia G. 1999. Mutations in ribosomal protein S19 gene and Diamond Blackfan anemia: wide variations in phenotypic expression. Blood 94:4294-4306.

Xu WB, Roufa DJ. 1996. The gene encoding human ribosomal protein S24 and tissue-specific expression of differentially spliced mRNAs. Gene 169:257-62.

Zhang Y, Wolf GW, Bhat K, Jin A, Allio T, Burkhart WA, Xiong Y. 2003. Ribosomal protein L11 negatively regulates oncoprotein MDM2 and mediates a p53-dependent ribosomal-stress checkpoint pathway. Mol Cell Biol 23:8902-12.

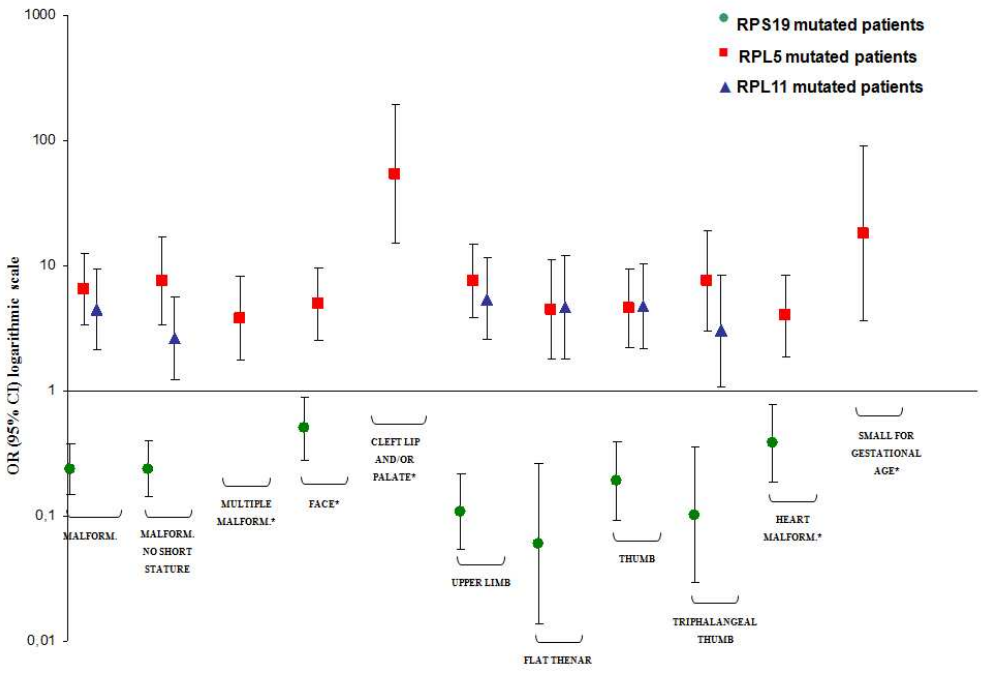
Zhang J, Harnpicharnchai P, Jakovljevic J, Tang L, Guo Y, Oeffinger M, Rout MP, Hiley SL, Hughes T, Woolford JL Jr. 2007. Assembly factors Rpf2 and Rrs1 recruit 5S rRNA and ribosomal proteins rpL5 and rpL11 into nascent ribosomes. Genes Dev 21:2580-92.

Zhang Y, Lu H. 2009. Signaling to p53: ribosomal proteins find their way. Cancer Cell 16:369-77.

Zhu Y, Poyurovsky MV, Li Y, Biderman L, Stahl J, Jacq X, Prives C. 2009. Ribosomal protein S7 is both a regulator and a substrate of MDM2. Mol Cell 35:316-26.

Figure Legends.

FIGURE 1. Malformation status of patients with *RPS19*, *RPL5* and *RPL11* mutations. Associations between malformations and RP gene mutations are assessed with odds ratio (OR) and 95% CI from logistic regression; OR are drawn on a logarithmic scale. (*) Non significative in *RPS19* and/or *RPL11*.



122x84mm (200 x 200 DPI)

Table 1. Newly reported mutations in *RPS19*, *RPL5*, *RPL11* and *RPS24*. Nucleotide numbering reflects cDNA numbering with +1 corresponding to the A of the ATG translation initiation codon in the reference sequence, according to journal guidelines (www.hgvs.org/mutnomen). The initiation codon is codon 1.

Mutated Gene	Patient (Gender)	Exon/Intron	cDNA Mutation	Predicted amino acid change	Mutation Type	Malformations	Growth Retardation	Steroid Response	Inheritance
RPS19	Ps19_1(F)	Ex 2	c.2T>A	p.Met?	Missense	na	na	na	familial
	Ps19_2(F)	Ex 2	c.10_13delGTTA	p.Val4LeufsX2	Deletion	none	no	yes	sporadic
	Ps19_3(F)	Ex 2	c.14delC	p.Thr5MetfsX2	Deletion	na	na	na	unknown
	Ps19_4(M)	Ex 2	c.28_29insT	p.Asn10IlefsX41	Insertion	na	na	na	sporadic
	Ps19_5(F)	Ex 2	c.34_47del	p.Gln12SerfsX34	Deletion	Dystrophy	na	na	unknown
	Ps19_6(M)	Ex 2	c.49G>C	p.Ala17Pro	Missense	na	na	na	sporadic
	Ps19_7(NA)	Ex 2	c.58G>C	p.Ala20Pro	Missense	na	na	na	unknown
	Ps19_8(F)	Ex 3	c.83T>G	p.Leu28Arg	Missense	none	no	na	unknown
	Ps19_9(M)	Ex 3	c.88delG	p.Val30SerfsX46	Deletion	Microcephaly, micro-retrognathy, hypertelorism, cafe au lait spots	na	na	familial
	Ps19_10(M)	Ex 3	c.93delC	p.Glu32AsnfsX44	Deletion	Thumb	no	no	unknown
	Ps19_11(M)	Ex 3	c.103dupG	p.Asp35GlyfsX16	Insertion	none	no	no	unknown
	Ps19_12(M)	Ex 3	c112A>T	p.Lys38X	Nonsense	none	no	no	sporadic
	Ps19_13(M)	Ex 3	c.156G>A	p.Trp52X	Nonsense	Low hairline, cafe au lait spots	na	na	unknown
	Ps19_14(M)	Ex 3	c.172G>C	p.Ala58Pro	Missense	none	yes	no	de novo
	Ps19_15(F)	Ex 4	c.178A>C	p.Thr60Pro	Missense	none	no	na	sporadic
	Ps19_16(M)	Ex 4	c.187_189insCAC	p.His63dup	Insertion	Flat nose, low hairline, mitral vale and tricuspid valve insufficiency	na	na	sporadic
	Ps19_17(NA)	Ex 4	c.195C>G	p.Tyr65X	Nonsense	na	na	na	unknown
	Ps19_18(NA)	Ex 4	c.195C>G	p.Tyr65X	Nonsense	na	na	na	unknown
	Ps19_19(F)	Ex 4	c.203_204insG	p.Gly69TrpfsX85	Insertion	na	na	na	sporadic
	Ps19_20(M)	Ex 4	c.212G>A	p.Gly71Glu	Missense	na	na	na	sporadic
	Ps19_21(F)	Ex 4	c.281G>T	p.Arg94Leu	Missense	none	na	na	sporadic
	Ps19_22(F)	Ex 4	c.284delG	p.Gly95AlafsX16	Deletion	ASD	na	na	unknown
	Ps19_23(M)	Ex 4	c.289_290insAGGC	p.Lys97ArgfsX58	Insertion	Dysplastic aortic valve	na	na	unknown
	Ps19_24(F)	Ex 4	c.296_297delTG	p.Val99GlyfsX54	Deletion	na	na	na	unknown
	Ps19_25(M)	Ex 4	c.301C>T	p.Arg101Cys	Missense	na	na	yes	unknown

	Ps19_26(F)	Ex 4	c.305G>C	p.Arg102Pro	Missense	none	no	na	unknown
	Ps19_27(M)	Ex 4	c.320T>G	p.Leu107Arg	Missense	na	na	na	unknown
	Ps19_28(F)	Ex 4	c.344delA	p.Lys115ArgfsX9	Deletion	na	na	na	familial
	mother (F)	Ex 4	c.344delA	p.Lys115ArgfsX9	Deletion	High palatine	na	na	unknown
	Ps19_29(NA)	Ex 4	c.356_357insG	p.Gly120ArgfsX34	Donor splice site	na	na	na	unknown
	Ps19_30(F)	Ex 5	c.372_373insA	p.Pro125ThrfsX29	Insertion	Hip subluxation on both sides	na	na	sporadic
	Ps19_31(F)	Ex 5	c.401_402insT	p.Ala135ArgfsX19	Insertion	na	na	na	unknown
	Ps19_32(F)	Ex 6	c.418delG	p.Ala140Leufs	Deletion	na	na	na	sporadic
	Ps19_33(M)	Int 1	c.-1G>C	p.0?	Acceptor splice site	Short stature	na	yes	familial
	Ps19_34(M)	Int 1	c.1-2A>T	p.0?	Acceptor splice site	none	no	na	familial
	brother (M)	Int 1	c.1-2A>T	p.0?	Acceptor splice site	none	no	na	familial
	Ps19_35(M)	Int 2	c.71+1G>C	p.0?	Donor splice site	na	na	na	de novo
	Ps19_36(M)	Int 2	c.72-1G>A	p.0?	Acceptor splice site	Macrocephaly, mental retardation	na	na	unknown
	Ps19_37(F)	Int 2	c.72-2A>C	p.0?	Acceptor splice site	Thumb	yes	yes	sporadic
	Ps19_38(M)	Int 3	c.172+1G>T	p.0?	Donor splice site	na	na	no	de novo
	Ps19_39(M)	Int 3	c.172+1G>T	p.0?	Donor splice site	Triphalangeal thumbs	yes	yes	sporadic
	Ps19_40(M)	Int 3	c.172+1G>C	p.0?	Donor splice site	na	na	no	unknown
	Ps19_41(F)	Int 3	c.173-2A>G	p.0?	Acceptor splice site	Low set ears	yes	no	de novo
	Ps19_42(F)	Int 3 / Ex 4	c.173-7_174del	p.0?	Deletion	na	na	na	unknown
	Ps19_43(M)	Int 4	c.356+1G>T	p.0?	Donor splice site	none	no	yes	sporadic
	Ps19_44(F)	Int 4	c.356+1_356+2delGTins12	p.0?	Donor splice site	na	yes	nd	unknown
	Ps19_45(M)	Int 5 / Ex 6	c.412-13_417del	p.0?	Deletion	none	no	na	de novo
RPL5	Pl5_1(F)	Ex 1	c.1A>G	p.Met1?	Missense	Triphalangeal thumb	na	na	sporadic
	Pl5_2(F)	Ex 1	c.2T>G	p.Met1Arg	Missense	Duplicated ureter	yes	yes	familial
	father (M)	Ex 1	c.2T>G	p.Met1Arg	Missense	Heart Murmur	yes	yes	familial
	Pl5_3(M)	Ex 2	c.48C>A	p.Tyr16X	Nonsense	Cleft palate, abnormal right thumb	yes	yes	de novo
	Pl5_4(F)	Ex 3	c.91delT	p.Tyr31MetfsX7	Deletion	Triphalangeal thumb	na	na	de novo
	Pl5_6(M)	Ex 3	c.172_173insA	p.Arg58LysfsX55	Insertion	Cleft lip and palate, triphalangeal thumb, short stature	na	na	de novo
	Pl5_7(F)	Ex 4	c.191_204ins14	p.Ile64LeufsX10	Insertion	na	na	na	de novo
	Pl5_8(F)	Ex 4	c.208G>T	p.Glu70X	Nonsense	na	na	na	sporadic

	Pl5_9(M)	Ex 4	c.283delT	p.Tyr95MetfsX31	Deletion	na	na	na	sporadic
	Pl5_10(M)	Ex 5	c.454delA	p.Arg152GlufsX12	Deletion	Dysmorphic face, VSD, cleft soft palate, triphalangeal thumbs, reflux of the left ureter	yes	yes	sporadic
	Pl5_11(M)	Ex 6	c.535C>T	p.Arg179X	Nonsense	Cleft lip, abnormality of bilateral second toe, aortic valve defect	yes	no	sporadic
	Pl5_12(F)	Ex 6	c.535C>T	p.Arg179X	Nonsense	na	na	na	sporadic
RPL11	Pl11_1(F)	Ex 2	c.100_101dupA	p.Thr34AsnfsX21	Insertion	VSD, ASD, abnormal thumbs	no	na	sporadic
	Pl11_2(M)	Ex 5	c.475_476ins11	p.Lys159ThrfsX39	Insertion	none	no	yes	sporadic
	Pl11_3(M)	Int 2	c.158-2A>C	p.0?	Acceptor splice site	none	yes	yes	sporadic
RPS24	Ps24_1(M)	Int 4	c.390+1G>A	p.0?	Donor splice site	na	na	na	sporadic

F: female; M: male; na: not available; VSD: ventricular septal defect; ASD: atrial septal defect.

Table 2. Pathogenetic point mutations in RP genes that are homologous to pseudogene sequences. Nucleotide numbering reflects cDNA numbering with +1 corresponding to the A of the ATG translation initiation codon in the reference sequence, according to journal guidelines (www.hgvs.org/mutnomen). The initiation codon is codon 1.

Gene name	DNA Mutation	Mutation Type	Exon/Intron	Gene sequence	Mutated sequence	Pseudogene sequence	Pseudogene name (locus)
RPS19	c.384_385delAA	Deletion	Ex 5	GGACA**AA**GAGAT	GGACA- -GAGAT	GGACA- -GAGAT	RPS19P2 (1p13.2)
	c.403G>A	Missense	Ex 5	GAATC**G**CCGGA	GAATC**A**CCGGA	GAATC**A**CCGGA	RPS19P2 (1p13.2)
	c.191T>C	Missense	Ex 4	GCACC**T**GTACC	GCACC**C**GTACC	GCACC**C**GTACC	RPS19P4 (5q11.2)
	c.166C>T	Nonsense	Ex 3	ACACG**C**GAGCT	ACACG**T**GAGCT	ACACG**T**GAGCT	RPS19P7 (10q11.21)
RPL5	c.535C>T	Nonsense	Ex 6	CCAAA**C**GATTC	CCAAA**T**GATTC	CCAAA**T**GATTC	RPL5P34 (22q13.2)*
RPL11	c.94_97delAGAC	Deletion	Ex 2	GAGAC**AGAC**TGACG	GAGAC----TGACG	GAGAC----TGACG	RPL11P5 (12q24.31)

** Note that this variation is carried also by other pseudogenes, but in a slighty different context (data not shown).*

Table 3. Clinical data relative to all DBA patients reported in the DBA Mutation Database.

Gene Name	Patients with Mutations*	Patients with Malformations	Malformations no Short Stature	Face	Cleft Lip and/or Palate	Upper Limb	Flat Thenar	Thumb	Triphalangeal Thumb	Heart Malf.	Genitourinary Anomalies	Mental Retardation	Small for Gestational Age	Multiple Malf.	GR	SR
RPS19	166	55	53	27	0	12	2	11	3	14	7	8	0	15	41	39
RPL5	50	42	41	24	21	28	10	18	12	15	3	1	7	14	6	19
RPL11	36	26	24	5	2	19	8	14	6	6	3	0	2	2	2	19
RPL35A	5	4	4	1	0	0	0	0	0	1	3	0	0	1	na	4
RPS26	10	3	3	1	1	0	0	0	0	0	2	0	0	1	na	4
RPS7	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
RPS10	4	1	1	1	0	0	0	0	0	0	0	0	0	0	na	3
RPS17	3	1	1	1	0	1	1	0	0	0	0	0	0	1	2	2
RPS24	10	3	3	0	0	1	1	0	0	1	1	0	0	1	1	5

GR: growth retardation; SR: steroid response. (*) Total number of patients whose clinical data were available.

Table 4. Summary of the pathogenic variants in DBA Mutation Database.

Type of Mutation	Gene										Patients*
	RPS19	**RPS26**	**RPS24**	**RPS17**	**RPS10**	**RPS7**	**RPL5**	**RPL11**	**RPL35A**	**Total**	
Missense	36	4	1	2	1	0	6	1	1	**52**	110
Nonsense	14	0	2	0	1	0	7	2	1	**27**	56
Small insertions and deletions	44	1	1	1	1	0	21	17	1	**87**	121
Splice site defects	23	3	2	0	0	1	5	6	0	**40**	53
Large deletions/ rearrangements	12	0	0	0	0	0	0	0	2	**14**	15
Total	**129**	**8**	**6**	**3**	**3**	**1**	**39**	**26**	**5**	**220**	**355**

(*) We have here considered the association with an individual patient irrespective to the fact that a mutation could recur in the same family.

SUPPLEMENTARY MATERIAL

METHODS

MUTATION DETECTION AND PATHOGENICITY ASSESSMENT

Mutation detection has been performed using automatic sequencing. For each group details can be found on their previous papers [Gazda et al., 2008; Cmejla at al., 2009; Quarello at al., 2010; Proust et al., 2003].

Generally, the complete coding sequence and exon-intron boundaries have been sequenced to eliminate the possibility of the presence of other mutations. Verification has been performed in keeping with the journal's requirements. In particular, all mutations have been confirmed on a second sample and on a second PCR product. In absence of biochemical data, pathogenicity of missense mutations have been established by at least two methods. In turn, the following methods were used: linkage to disease in a family, concurrent appearance of the phenotype with a *de novo* mutation, or determination that the mutation is absent among at least 50 normal individuals (100 alleles) or involves a highly conserved amino acid. In some cases the Polyphen algorithm has been used. Otherwise pathogenicity is reported as uncertain in the Database.

FIGURES

SUPPLEMENTAL FIGURE S1. The main page of the RPS19 mutation database.

SUPPLEMENTAL FIGURE S2. Schematic drawings showing the location of the pathogenic variants in relation to the corresponding gene structure.

SUPPLEMENTAL FIGURE S1.

LOVD Leiden Open Variation Database

Diamond-Blackfan Anemia
ribosomal protein S19 (RPS19)
Curators: I. Boria and U. Ramenghi

Home | Variants | Submitters | Submit | Configuration | Setup | Documentation

RPS19 homepage | Switch gene

LOVD Gene homepage

General information	
Gene name	ribosomal protein S19
Gene symbol	**RPS19**
Chromosome Location	19q13.2
Database location	www.dbagenes.unito.it
Curator	I. Boria and U. Ramenghi
Database reference for citations	Boria et al (2008). A new database for ribosomal protein genes which are mutated in Diamond-Blackfan Anemia. Hum. Mutat.29(11), E263-70
PubMed references	View all (unique) PubMed references in the RPS19 database
Date of creation	November 12, 2007
Last update	June 11, 2010
Version	**RPS19 100611**
Add sequence variant	Submit a sequence variant
First time submitters	Register here
Reference sequence	coding DNA reference sequence for describing sequence variants
GenBank reference	NM_001022.3
Total number of unique DNA variants reported	**129**
Total number of individuals with variant(s)	**219**
Total number of variants reported	**219**
Subscribe to updates of this gene	
NOTE	The RPS19 gene comprises 6 exons and spans 11 Kb. The first exon (372 bp) is included in the 5' UTR region, while the other five (435 bp) encode for a 145 amino acid protein (MW 16 kDa). The predicted size of the whole RPS19 primary transcript, including the polyA stretch, is 872 bp. RPS19 has 3 annotated pseudogenes. Link to the location of the pathogenic variants in relation to the gene Link to the location of the pathogenic variants in relation to the protein structure

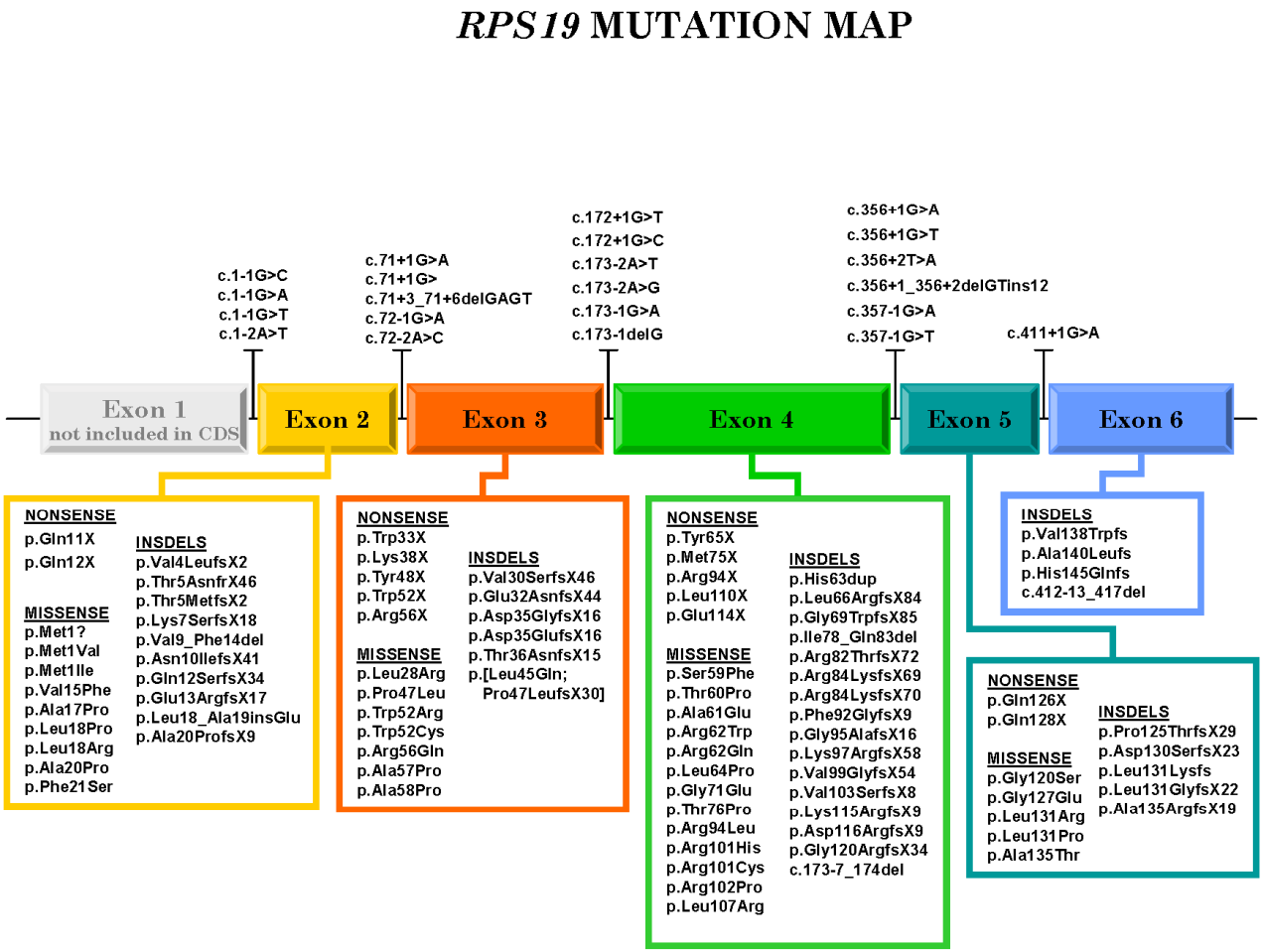
Graphical displays and utilities	
Summary tables	Summary of all sequence variants in the RPS19 database, sorted by type of variant (with graphical displays and statistics)

Sequence variant tables	
Unique sequence variants	Listing of all unique sequence variants in the RPS19 database, without patient data
Complete sequence variant listing	Listing of all sequence variants in the RPS19 database
Variants with no known pathogenicity	Listing of all RPS19 variants reported to have no noticeable phenotypic effect (note: excluding variants of unknown effect)

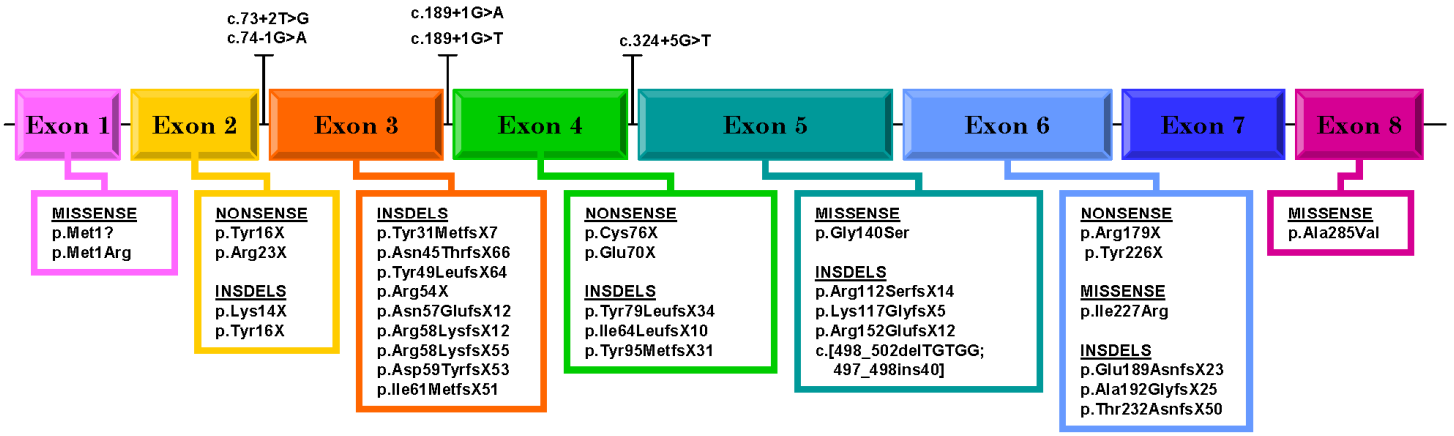
Search the database	
By type of variant	View all sequence variants of a certain type
Simple search	Query the database by selecting the most important variables (exon number, type of variant, disease phenotype)
Advanced search	Query the database by selecting a combination of variables
Based on patient origin	View all variants based on your patient origin search terms
Search through hidden entries	Find the number of variant entries in the database (including hidden entries) matching your search terms.

Links to other resources	
External link	dbSNP
Entrez Gene	6223
OMIM - Gene	603474
OMIM - Disease	105650 (DBA1)
HGMD	RPS19

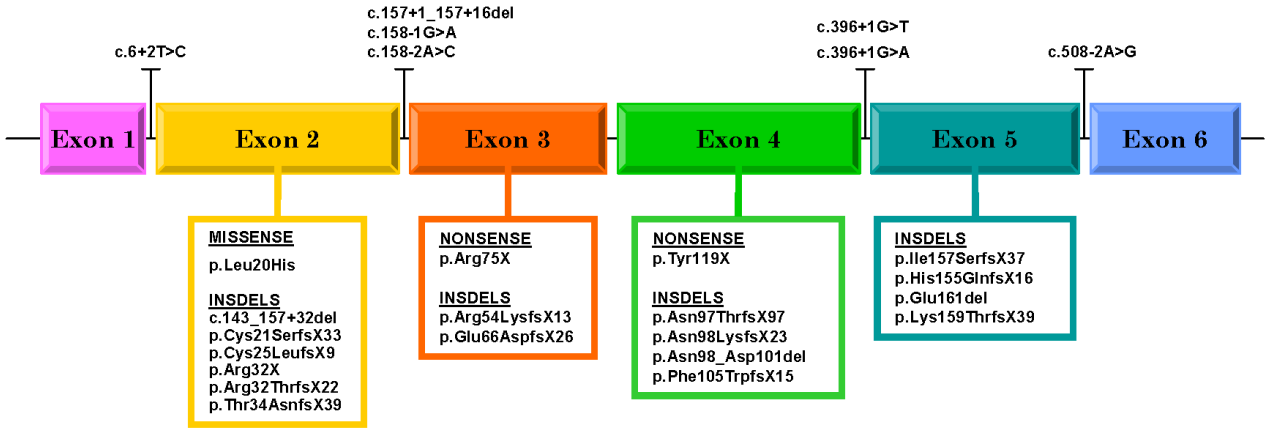
SUPPLEMENTAL FIGURE S2.



RPL5 MUTATION MAP

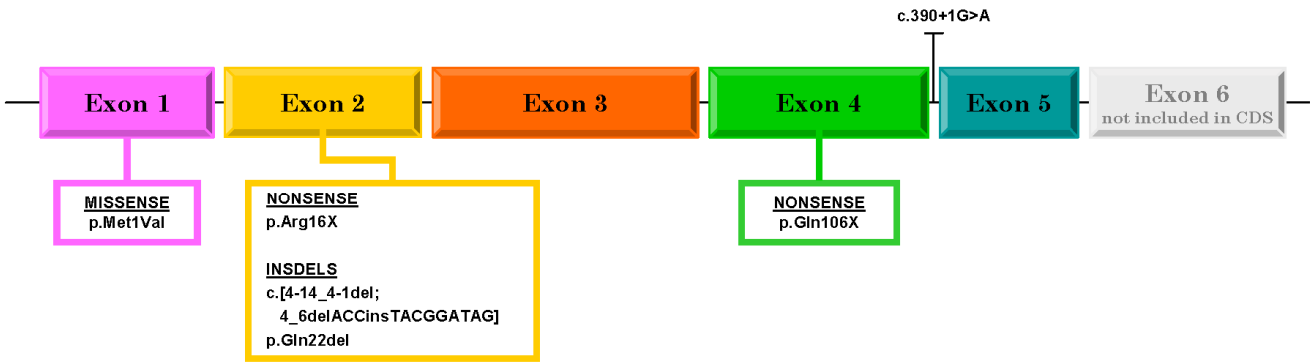


RPL11 MUTATION MAP

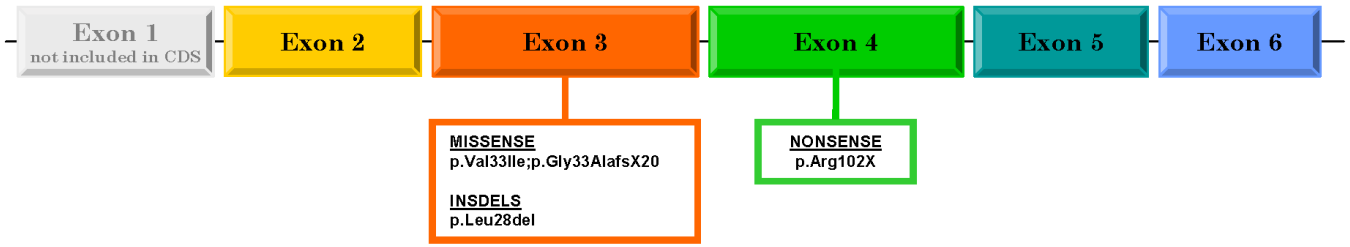


RPS24 MUTATION MAP

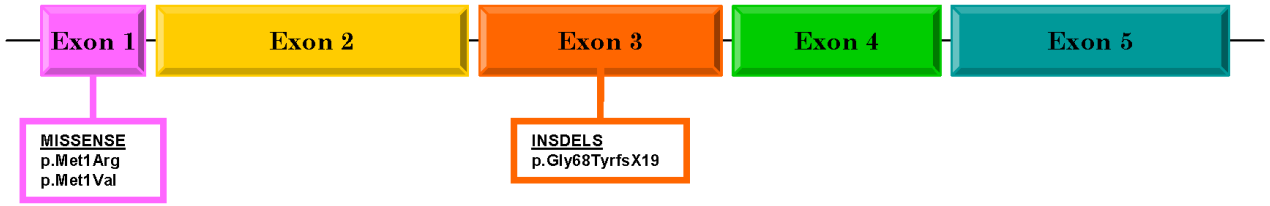
NM_033022.3, (variant a)



RPL35A MUTATION MAP

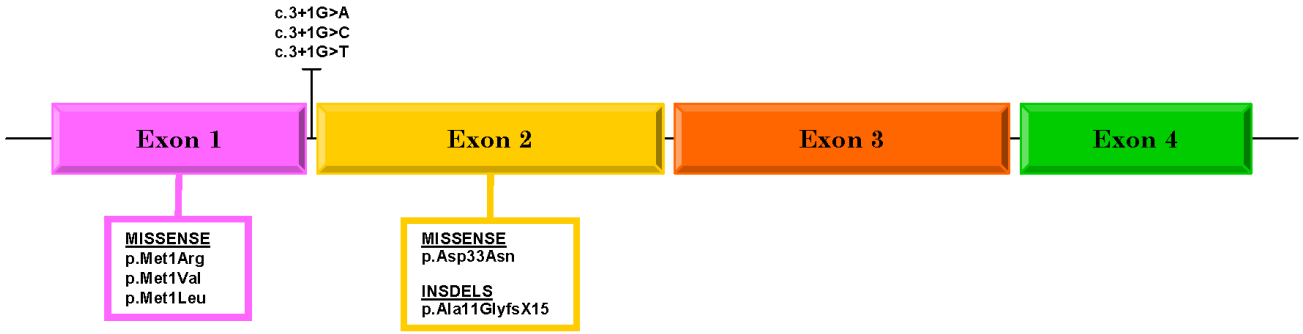


RPS17 MUTATION MAP



RPS7 MUTATION MAP

RPS26 MUTATION MAP



RPS10 MUTATION MAP